



**PATENT APPLICATION**  
**Docket No. 356830.00300**

Former Docket No. 18810-80364

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

JULIA LJUBIMOVA et al.

Serial No.: 09/741,550

Filed: December 19, 2000

For: USING OVEREXPRESSION OF  
LAMININ ALPHA 4 SUBUNIT AS A  
DIAGNOSTIC AND PROGNOSTIC  
INDICATOR OF MALIGNANT TUMORS

Examiner: J. A. Goldberg

Art Unit: 1634

**SECOND DECLARATION OF JULIA LJUBIMOVA**  
**UNDER RULE 132 (37 CFR 1.132)**

I, JULIA LJUBIMOVA, declare as follows:

1. I am a named inventor on the present application and a citizen of the United States residing at 11419 Dona Pegita Dr., Studio City, California 91604

2. I obtained my M.D. degree from Kiev Medical University, Kiev, Ukraine in 1983 and a Ph.D. degree in Oncology-Pathology from the Kavetsky Institute for Oncology Problems, Academy of Sciences of Ukraine, Kiev, Ukraine. My thesis was entitled "Molecular peculiarities of DNA in malignant melanomas and benign nevi of human skin".

3. I have held positions of Staff Research Associate in the Department of Reproductive Medicine at the University of California San Diego and Research Scientist in the Department of Surgery and Neurosurgical Institute at Cedars-Sinai Medical Center, Los Angeles, California. I have also held appointments as an Adjunct Assistant Professor in the Department of Surgery at the University of California Los Angeles.

4. My current title is Director, Molecular Oncology Maxine Dunitz Neurosurgical Institute, Cedars-Sinai Medical Center. I also serve as a Clinical Assistant Professor in the Department of Neurosurgery at the University of California Irvine.

5. I have over twenty published scientific publications and have given numerous lectures and scientific presentations on cancer and related subjects.

6. I have a great deal of experience with malignancies both at an experimental and clinical level. During the studies that led to the above-named patent application I realized that  $\alpha 4$  laminin undoubtedly had significance in many, if not all, malignancies partially because of its relationship to vasculature in malignancies. Complete clinical studies are difficult and time consuming. However, prior to filing the above named application I was able to perform preliminary tests on malignant tumors not of brain origin. Although human breast cancer appears quite different from brain cancer, I discovered that like brain malignancies, malignant tumors of the breast overexpress  $\alpha 4$  laminin.

7. Since the filing of the present patent application I have expanded my observations on malignant breast tumors into a full study that parallels the instant patent application. As I show in the data presented below, the results for malignant breast tumors are very similar to those for malignant brain tumors. However, the picture with breast tissue is somewhat more complex because of the presence of

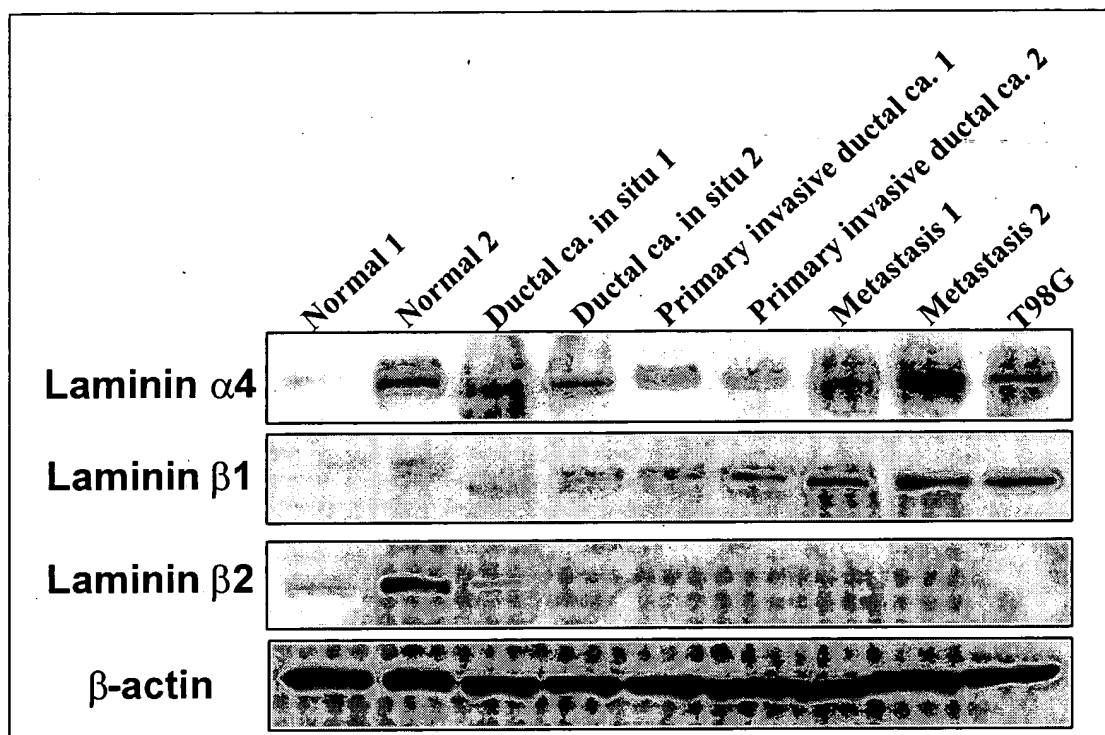
several different tissue types including ductal cells within a breast biopsy whereas a brain biopsy lacks the same diversity of tissues. As explained and demonstrated in paragraphs 12 and 13, below, there is some  $\alpha 4$  laminin expression in normal ductal tissue but not in normal breast vasculature. There is significant  $\alpha 4$  laminin expression in vessels of breast cancers—this corresponds to the situation in brain cancer. Because breast tissue and brain tissue are inherently dissimilar tissues, the similarity in  $\alpha 4$  laminin expression supports the theory that such expression is a marker of malignancy and increases the invasiveness of the malignancy—apparently through some effect on the vasculature.

8. To demonstrate the present invention with breast malignancies A total of 45 human breast tissue samples were used for Western blot analysis and immunohistochemistry, including 14 normal breast tissues, 4 ductal carcinoma *in situ* (DCIS), 23 primary invasive ductal carcinoma of breast and 3 brain metastases of breast cancer.

9. For Western Blot analysis twenty-eight of the tissue samples were used: 10 normal breast tissues, 4 DCIS, 11 primary invasive ductal carcinomas and the 3 brain metastasis of breast cancer. Tissue samples were snap-frozen in liquid nitrogen by a pathologist immediately after surgery. Proteins were separated using 10% Tris-Glysin SDS-PAGE (Invitrogen, Carlsbad, CA) under reducing conditions. Lysates of human glioma T98G, known to express Laminin-8 (Ln-8) were used as a positive control. The gels were blotted onto nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membranes were probed with monoclonal antibodies followed by chemiluminescent detection using the Immun-Star<sup>TM</sup> AP kit with alkaline phosphatase-conjugated secondary antibodies (BIO-RAD, Hercules, CA). Antibodies were used to the Ln  $\alpha 4$  chain (mAb 8B12)<sup>15</sup>,  $\beta 1$  chain (rAb LT3)(upstate, Lake Placid, NY) and  $\beta 2$  (clone C4)(LAB VISION, Fremont, CA). Antibody to  $\beta$ -actin (SIGMA, St. Louis, MO) was used to control for equal loading of

gel lanes. The intensities of the bands of interest were expressed relative to the  $\beta$ -actin band intensities for the same specimens, using the AlphaImager<sup>TM</sup> 2000 densitometer (AlphaInnotech, Inc., San Leandro, CA). Background was subtracted from each reading before determining the intensity ratios.

10. We compared the expression of  $\alpha 4$ ,  $\beta 1$  and  $\beta 2$  chains of laminin using Western blot analysis. The expression of the  $\alpha 4$  chain of laminin that is one of the constituents of Ln-8 is various in tumor tissues and also in corresponding normal tissues (Fig. 1). However, the expressions of  $\beta 2$  chains are higher in normal tissues than in cancerous tissues. Furthermore, the intensity patterns of  $\beta 2$  expressions in normal tissues are similar to those of  $\alpha 4$  expression. On the other hand, the expression of  $\beta 2$  is lower or absent in the tissues of primary tumor and metastasis. These results tend to show that the occasional strong expression of  $\alpha 4$  in normal tissues correspond to Laminin-9 (Ln-9) but not Ln-8. It is believed that variability is due to the mix of tissue types included in a given biopsy. In contrast, the expression level of  $\beta 1$  chains was higher in tumor tissues and the expression levels are ranked in tumor grade dependent manner. Though the expression of  $\beta 1$  chains of laminin is present in normal breast tissues, the level of expression is lower than in cancerous tissues. To take the expression of  $\alpha 4$  and  $\beta 2$  into consideration, the expression pattern of the combination with  $\alpha 4$  and  $\beta 1$  is more predominant in cancerous tissues than normal tissue. Specifically in brain metastasis of breast cancer, the expression of the combination with  $\alpha 4$  and  $\beta 1$  is higher than in those of primary breast or normal breast tissues. However, from the viewpoint of histology, multiple types of cell components are included in the breast tissues. Therefore, to clarify the localization of the each component of laminin chains and to identify the laminin complex, we used immunohistochemistry.



**Figure 1.** Western blot analysis of normal breast tissues, ductal carcinomas in situ, invasive ductal carcinoma tissues and brain metastases from the primary breast cancer for Laminin  $\alpha 4$ ,  $\beta 1$  and  $\beta 2$  chains (typical results of 8 / 28 samples are shown). The expression of Laminin  $\alpha 4$  chain, a constituent of Laminin-8, varies in tumor tissues and also in corresponding normal tissues. However, the expression of  $\beta 2$  chain, a constituent of Laminin-9, is relatively high in normal tissues, but is very low or non-existent in breast cancer tissues. The levels of  $\beta 2$  expressions in each normal tissue are similar to those of  $\alpha 4$  expressions. These results may show that any strong expression of  $\alpha 4$  in normal tissues corresponds to Laminin-9 but not to Laminin-8. In contrast, the expression of Laminin  $\beta 1$  chain, another constituent of Laminin-8, is high in brain metastases and primary invasive ductal carcinoma, but the expression decreases in ductal carcinomas in situ and is very low in normal tissues. Laminin  $\alpha 4$  chain migrates at 200 kDa; Laminin  $\beta 1$  chain, at 230 kDa; Laminin  $\beta 2$  chain, at 190 kDa;  $\beta$ -actin, at 47kDa. T98 glioblastoma cell line that only expresses chains of Laminin-8 ( $\alpha 4$  and  $\beta 1$ ) is used as a positive control.

11. Thirty-eight tissue samples were used for immunocytochemical analysis: 14 normal breast tissues, 4 DCIS, 15 primary invasive ductal carcinoma and 3 breast cancer metastases in the brain. Tissue samples were snap-frozen in liquid nitrogen by a pathologist immediately after surgery and embedded in OCT

compound, and 8-mm sections were cut on a cryostat. Indirect immunofluorescence, photography, and routine negative controls were obtained. Polyclonal and monoclonal antibodies were used against laminin chains  $\alpha 4$  (clone 1F9),  $\beta 1$  (clone LT3), and  $\beta 2$  (clone C4). Rabbit polyclonal antibodies against laminin  $\alpha 4$  chains (LG1-3) were also used. The secondary cross-species absorbed fluorescein- and rhodamine-conjugated donkey anti-mouse, anti-rat, and anti-rabbit antibodies were from Chemicon International (Temecula, CA). The polyclonal antibodies against human von Willebrand factor (Sigma, St. Louis, MO) were used for endothelial cell detection. Monoclonal antibodies against cytokeratin 8/18 (biomeda, Foster, CA) were used for epithelial cell detection. Monoclonal antibodies were used as straight hybridoma supernatants or at 10-20 mg/ml when purified, and polyclonal antibodies were used at 20-30 mg/ml.

12. The expression of each of the laminin chains was investigated using 36 clinical samples (normal 14, in situ 4, invasive breast cancer 15, brain metastasis 3) to determine  $\alpha 4$  and  $\beta 1$  co-localization. The expressions of  $\alpha 4$  chains in invasive ductal carcinoma and brain metastatic tissues were always co-localized with  $\beta 1$  chains. While, the expressions of  $\alpha 4$  chains were co-localized mainly with  $\beta 2$  chains in normal breast tissues. These data indicate that Ln-8 ( $\alpha 4\beta 1\gamma 1$ ) expresses in breast tumor tissue but Ln-9 ( $\alpha 4\beta 2\gamma 1$ ) expresses in normal tissue. As shown in Table 1, Ln-8 expressed 93.3% primary invasive breast cancer tissues and 100% brain metastasis. The Ln-8 expressing tissues were decreased in DCIS and we detect low signal of Ln-8 in normal tissue (Table 1). In contrary, the expressions of Ln-9 were detected in all normal tissues including 92.8% of vessels BMs (basement membranes), 100% of mammary duct's BMs (Table 1) and adipose tissues (data not shown). The reason why the expression of Ln-9 were variable in normal tissues seems to be because of the large variety of histological components in normal tissues including mammary ducts, vessels, connective tissues and adipose tissues. The BMs of mammary ducts that are involving Ln-9 partially disappeared in DCIS

and are absent in invasive ductal carcinoma and metastatic tissues. But only 6.7% in primary invasive breast tumor tissue and none of brain metastasis expressed Ln-9 without Ln-8 at the BMs of vessels (Table 1).

Table 1.

Histological diagnosis	Number of cases <i>n</i>	Laminin-8 <i>n</i> (%)	Laminin-8/-9 <i>n</i> (%)	Laminin-9 <i>n</i> (%)
Normal breast tissue	14	1 (7.1%)	1 (7.1%)	12 (85.7%)
Ductal carcinoma in situ	4	1 (25%)	2 (50%)	1 (25%)
Invasive ductal carcinoma	15	11 (73.3%)	3 (20%)	1 (6.7%)
Metastases	3	3 (100%)	0	0

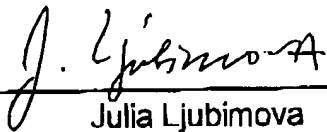
13. To identify the localization of Ln-8 in the breast cancer tissues, we co-stained  $\alpha 4$  or  $\beta 1$  chains with endothelial marker (von Willebrand factor) or epithelial marker (Cytokeratine 8/18). The  $\alpha 4$  or  $\beta 1$  chains were co-localized with the von Willbrand factor (VWF) in invasive ductal carcinoma and metastatic tissues but were not co-localized with cytotkeratine. However, the  $\beta 2$  co-localized with basement membranes of mammary ducts and the vessels in normal tissues. In addition, though we also detect low expression of  $\beta 1$  in normal vessels of some tissue samples, the expression was very low in comparison with the expression of that in cancerous tissues. In DCIS, the expressions of both Ln-8 and Ln-9 were seen at the basement membranes of microvessels independently. These data suggest that the switch from Ln-9 to Ln-8 in the process of breast cancer progression starts at early phase of carcinogenesis. Moreover, the results clearly shows Ln-8 is dominant in the tumor microvessel basement membrane in breast cancer tissues, while Ln-9 is predominant the normal breast vessel basement membranes.

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## Ljubimova Rule 132 Declaration

I hereby declare under penalty of perjury that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed this 28<sup>th</sup> day of July 2004 at Los Angeles, California .

  
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Julia Ljubimova

07/28/2004



I hereby declare under penalty of perjury that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed this 28<sup>th</sup> day of July 2004 at Los Angeles, California .

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Julia Ljubimova



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☐ 1: Z99289. Human DNA sequenc...[gi:3168992]

LOCUS HS142L7 190778 bp DNA linear PRI 04-MAR-2003  
 DEFINITION Human DNA sequence from clone RP1-142L7 on chromosome 6q21,  
 complete sequence.

ACCESSION Z99289

VERSION Z99289.1 GI:3168992

KEYWORDS HTG.

SOURCE Homo sapiens (human)

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
 Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 190778)

AUTHORS Tubby, B.

TITLE Direct Submission

JOURNAL Submitted (04-MAR-2003) Wellcome Trust Sanger Institute, Hinxton,  
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COMMENT On May 30, 1998 this sequence version replaced gi:2578058.

----- Genome Center

Center: Wellcome Trust Sanger Institute

Center code: SC

Web site: <http://www.sanger.ac.uk>

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During sequence assembly data is compared from overlapping clones.  
 Where differences are found these are annotated as variations  
 together with a note of the overlapping clone name. Note that the  
 variation annotation may not be found in the sequence submission  
 corresponding to the overlapping clone, as we submit sequences with  
 only a small overlap as described above.

This sequence was finished as follows unless otherwise noted: all  
 regions were either double-stranded or sequenced with an alternate  
 chemistry or covered by high quality data (i.e., phred quality >= 30);  
 an attempt was made to resolve all sequencing problems, such  
 as compressions and repeats; all regions were covered by at least  
 one plasmid subclone or more than one M13 subclone; and the  
 assembly was confirmed by restriction digest, except on the rare  
 occasion of the clone being a YAC.

The following abbreviations are used to associate primary accession  
 numbers given in the feature table with their source databases:

Em:, EMBL; Sw:, SWISSPROT; Tr:, TREMBL; Wp:, WORMPEP; Information  
 on the WORMPEP database can be found at

[http://www.sanger.ac.uk/Projects/C\\_elegans/wormpep](http://www.sanger.ac.uk/Projects/C_elegans/wormpep) This sequence

was generated from part of bacterial clone contigs of human  
 chromosome 6, constructed by the Sanger Centre Chromosome 6 Mapping  
 Group. Further information can be found at

<http://www.sanger.ac.uk/HGP/Chr6>

RP1-142L7 is from the library RPCI-1 constructed by the group of  
 Pieter de Jong. For further details see

<http://www.chori.org/bacpac/home.htm>